# 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol, a metabolite of ginsenoside Rb1, enhances the production of hyaluronic acid through the activation of ERK and Akt mediated by Src tyrosin kinase in human keratinocytes

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Received August 5, 2014; Accepted January 29, 2015

DOI: 10.3892/ijmm.2015.2121

**Abstract.** The aim of the present study was to determine the mechanisms through which 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (20GPPD) promotes the production of hyaluronic acid (HA) in human keratinocytes. 20GPPD is the primary bioactive metabolite of Rb1, a major ginsenoside found in ginseng (*Panax ginseng*). We sought to elucidate the underlying mechanisms behind the 20GPPD-induced production of HA. We found that 20GPPD induced an increase in HA production by elevating hyaluronan synthase 2 (HAS2) expression in human keratinocytes. The phosphorylation of extracellular signal-regulated kinase (ERK) and Akt was also enhanced by 20GPPD in a dose-dependent manner. The pharmacological inhibition of ERK (using U0126) or Akt (using LY294002) suppressed the 20GPPD-induced expression of HAS2, whereas treatment with an epidermal growth factor receptor (EGFR) inhibitor (AG1478) or an intracellular $Ca^{2+}$ chelator (BAPTA/AM) did not exert any observable effects. The increased Src phosphorylation was also confirmed following treatment with 20GPPD in the human keratinocytes. Following pre-treatment with the Src inhibitor, PP2, both HA production and HAS2 expression were attenuated. Furthermore, the 20GPPD-enhanced ERK and Akt signaling decreased following treatment with PP2. Taken together, our results suggest that Src kinase plays a critical role in the 20GPPD-induced production of HA by acting as an upstream modulator of ERK and Akt activity in human keratinocytes.

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*Key words:* 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol, hyaluronic acid, hyaluronan synthase 2, *Panax ginseng*, Src tyrosine kinase

## Introduction

Ginseng (*Panax ginseng*) has historically been used as an herbal medicine throughout Asia and is now commonly accessible worldwide. Following ingestion, a bioactive component of ginseng known as ginsenoside Rb1 is converted into 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (20GPPD) (Fig. 1A) by gastric fluids and the intestinal microflora (1). Previous studies have demonstrated the beneficial effects of ginsenosides and their metabolites on skin health, such as their wound healing (2,3) and anti-aging effects (4,5), as well as protective effects against untraviolet irradiation (6-8). Pharmacologically, 20GPPD has anti-inflammatory properties (9), anti-aging effects and promotes skin wound healing (5,10). Previously, our group demonstrated that 20GPPD exerts anticancer effects by inducing colon cancer cell apoptosis (11). It has also been reported that 20GPPD increases hyaluronan synthase (HAS)2 expression in human keratinocytes, although the mechanisms responsible are not yet fully understood.

Hyaluronic acid (HA) is a glycosaminoglycan composed of D-glucuronic acid and *N*-acetyl-D-glucosamine and functions as a major component of the vitreous body, joint fluids (12), and skin (13). HA plays a role in the proliferation, differentiation and migration of specific cell types (14), and functions through an interaction with cell-surface receptors (15). Approximately 7-8 g of HA are present in an average human body, and half of this amount is found in the skin. HA has the capacity to retain large amounts of water, and thus plays an important role in regulating physiological water balance and osmotic pressure (16). HA content within skin decreases with age (17), and therefore, the upregulation of HA levels may help maintain skin homeostasis during aging.

HA is synthesized in the plasma membrane by HAS1, 2 and 3 (18). Each synthase has distinct encoding sites, producing HA structures of different lengths and with distribution in different tissues during mouse development (19,20). In humans, HAS2 is present in normal tissues, while HAS3 is more commonly expressed in tumor cells or during inflammation (21). The genetic ablation of HAS2 causes embryonic lethality in mouse models, whereas the knockdown of HAS1 and HAS3 knockouts does lead to the development of any observable phenotype (19). In aged human skin, HAS2 gene expression is frequently decreased in the epidermis and dermis (17).

HAS2 is regulated by growth factors and cytokines. Epidermal growth factor (EGF) induces HA synthesis by increasing HAS2 and HAS3 expression (22), while platelet-derived growth factor (PDGF)-BB stimulates HAS2 expression through the activation of phosphatidylinositol 3-kinase (PI3K) and mitogen-activated/extracellular signal-regulated kinase (MEK) (23). Src is a non-receptor tyrosine kinase that plays critical roles in receptor signaling and cellular communication (24). Src is expressed in many cell types, including skin cells, and activates cell proliferation and migration (25). Src is activated by cell adhesion to the extracellular matrix (ECM) and growth factors (26), and subsequently regulates the activation of extracellular signal-regulated kinase (ERK), mitogen-activated protein kinases (MAPK) and PI3K signaling (24), which play a critical role in the PDGF-BB-induced production of HA in human dermal fibroblasts (23).

In the present study, we demonstrate that 20GPPD induces the production of HA through the stimulation of the Src/ERK and Akt signaling pathways in human keratinocytes.

## Materials and methods

*Materials*. 20GPPD was purchased from the Ambo Institute (Daejeon, Korea). Dulbecco's modified Eagle's medium (DMEM) was obtained from HyClone (Logan, UT, USA). Fetal bovine serum (FBS) and anti-β-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against HAS2 (sc-365263), phosphorylated ERK1/2 (E-4; sc-7383), and total ERK1 (K-23; sc-94) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against phosphorylated Src (Tyr416; 2101), Akt (Ser473; 9271), and total Akt (9272) were purchased from Cell Signaling Technology (Beverly, MA, USA). U0126 (ERK inhibitor) was obtained from Tocris Bioscience (Ellisville, MO, USA). LY294002 (Akt inhibitor) was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). AG1478 (EGFR inhibitor), BAPTA-AM (intracellular $Ca^{2+}$ chelator) and PP2 (Src inhibitor) were purchased from Calbiochem (Darmstadt, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder was purchased from USB Corp. (Cleveland, OH, USA). Penicillin/streptomycin mix was purchased from Gibco-BRL (Grand Island, NY, USA). The protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA, USA).

*Cell culture and MTT assay*. Human keratinocytes (HaCaT; kindly provided by Dr Zigang Dong, Hormel Institute, Univerisity of Minnesota, Minneapolis, MN, USA) were cultured at 37˚C in a 5% $CO_2$ atmosphere in DMEM supplemented with 10% FBS, 2 mM L-glutamine and penicillin/streptomycin. Cell cytotoxicity was measured by MTT assay. The cells were cultured in 96-well plates at a density of $2x10^3$ cells/well, and incubated at 37˚C in a 5% $CO_2$ atmosphere prior to serum deprivation for 24 h. Various concentrations of 20GPPD were added to the wells for 22 h. Following 2 h of incubation with 20 $\mu$l of MTT solution, the medium was removed. Dimethylsulfoxide (DMSO; 200 $\mu$l) was added to each well to dissolve the formazan crystals. Absorbance at 570 nm was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

*Enzyme-linked immunosorbent assay (ELISA)*. The HaCaT cells were grown to 50% confluence in 6-well plates in DMEM supplemented with 10% FBS. After 24 h of starvation with serum-free DMEM, the medium was removed. The wells were washed with serum-free DMEM to completely remove the HA that had accumulated during cell growth. Among the 6 wells, 2 wells were selected for PP2 pre-treatment for 1 h and the cells were then treated with the indicated concentrations of 20GPPD. The other 3 wells were treated with 20GPPD at various concentrations without PP2 pre-treatment. Following 3 h of incubation, the medium was collected and centrifuged at 1,100 x g for 3 min. The HA concentration in the supernatant was analyzed using an ELISA kit (Corgenix, Inc., Broomfield, CO, USA). ELISA was performed as per the manufacturer's instructions.

*Immunoblot analysis*. HaCaT cells were cultured at a density of $2x10^3$ cells for 48 h, prior to serum deprivation for an additional 24 h. The cells were treated with 20GPPD at the indicated concentrations. In order to determine the effects of 20GPPD on HAS2 expression, the cells were incubated with 20GPPD for 3 h. The effect of 20GPPD on ERK, Akt and Src phosphorylation was evaluated after 1 h of 20GPPD treatment. The cells were collected using lysis buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10% glycerol and a protease inhibitor cocktail tablet] which was prepared manually. Protein concentrations were measured using a dye-binding protein assay kit, according to the manufacturer's instructions (Bio-Rad Laboratories). Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Millipore Corp., Bedford, MA, USA). The membranes were blocked in 5% skim milk for 2 h and incubated overnight at 4˚C with primary antibodies. Subsequently, the membranes were incubated with HRP-conjugated secondary antibodies, and the antibody-bound proteins were

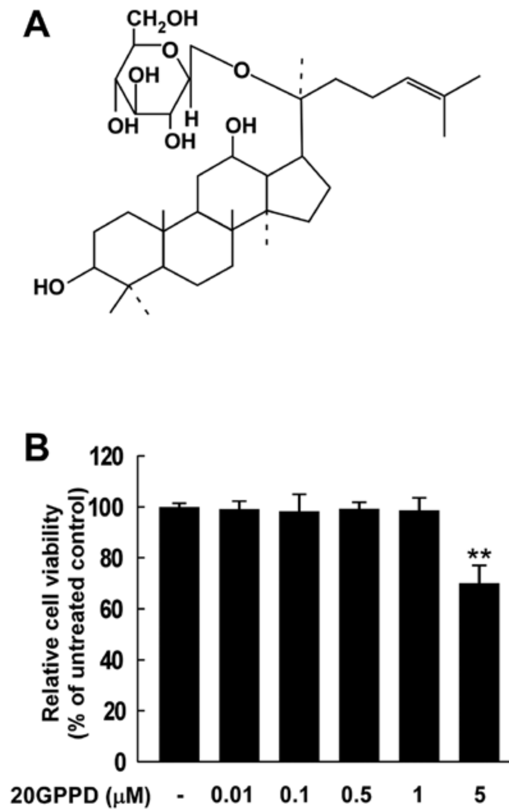


Figure 1. Effect of 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (20GPPD) on HaCaT cell viability. (A) Chemical structure of 20GPPD. (B) Viability of HaCaT cells treated with up to 1 µM 20GPPD. Following serum deprivation for 24 h, the cells were treated with different doses of 20GPPD for 24 h. The viability of the HaCaT cells was evaluated by MTT assay as described in the Materials and methods. Data represent the means ± SD. **P<0.01 vs. the untreated control.

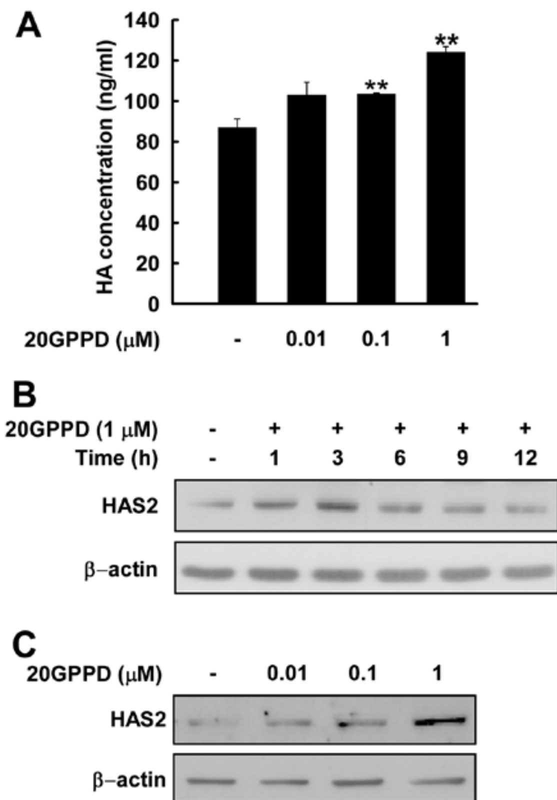


Figure 2. 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (20GPPD) increases the production of hyaluronic acid (HA) and hyaluronan synthase 2 (HAS2) expression in HaCaT cells. (A) 20GPPD increased HA production. Cells were starved in serum-free medium and treated with the indicated concentrations of 20GPPD for 3 h. HA concentrations were determined by enzyme-linked immunosorbent assay (ELISA). Data represent the means ± SD. **P<0.01 vs. the untreated control. (B and C) 20GPPD induced HAS2 expression in HaCaT cells. (B) Cells were starved in serum-free medium and treated with 20GPPD (1 µM) for indicated time periods of time. (C) Cells were starved in serum-free medium and treated with the indicated concentrations of 20GPPD for 3 h. The level of HAS2 and β-actin expression was determined by immunoblot analysis as described in the Materials and methods.

detected using a chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

*Statistical analysis*. Data are expressed as the means ± standard deviation (SD), and one-way ANOVA was used for single group comparisons. A value of P<0.05 was used as the criterion for statistical significance.

## Results

*20GPPD increases HA production in HaCaT cells*. A previous study reported that 20GPPD increased HAS2 mRNA expression in HaCaT cells (10). Since HAS2 is a critical enzyme in the production of HA, we sought to investigate the effects of 20GPPD on HA production. The non-cytotoxic concentration range of 20GPPD was first determined (Fig. 1B). Treatment with 20GPPD (0.01-1 µM) increased the production of HA in the HaCaT cells in a dose-dependent manner (Fig. 2A). We further monitored the changes in HAS2 expression in the cells following 1, 3, 6, 9 and 12 h of treatment with 20GPPD. The maximum expression of HAS2 was observed after 3 h of treatment with 20GPPD, and it gradually decreased back to the basal level over the time course of 12 h (Fig. 2B). In accordance with the HA production pattern, the expression of HAS2 was observed to be elevated by 20GPPD in a dose-dependent manner (Fig. 2C). These results demonstrate that 20GPPD induces the production of HA in a dose-dependent manner, and that this is associated with the increased expression of HAS2 in the presence of 20GPPD.

*20GPPD induces ERK and Akt phosphorylation in HaCaT cells*. It has previously been demonstrated that HAS2 expression is regulated by the MAPK and PI3K/Akt signaling pathways (23). Blocking p38 and MEK signaling has been shown to inhibit HAS2 expression by up to 90 and 40%, respectively, whereas the inhibition of JNK has no such effect (27). We therefore sought to determine the effects of 20GPPD on the phosphorylation of ERK and Akt. 20GPPD enhanced the phosphorylation of ERK and Akt in a dose-dependent manner, and these phosphorylation levels peaked at 0.5 and 1 h of 20GPPD treatment, respectively (Fig. 3). To examine whether these activated signaling pathways are associated with the 20GPPD-induced production of HA, pharmacological inhibitors of ERK and Akt (U0126 and LY294002, respectively) were

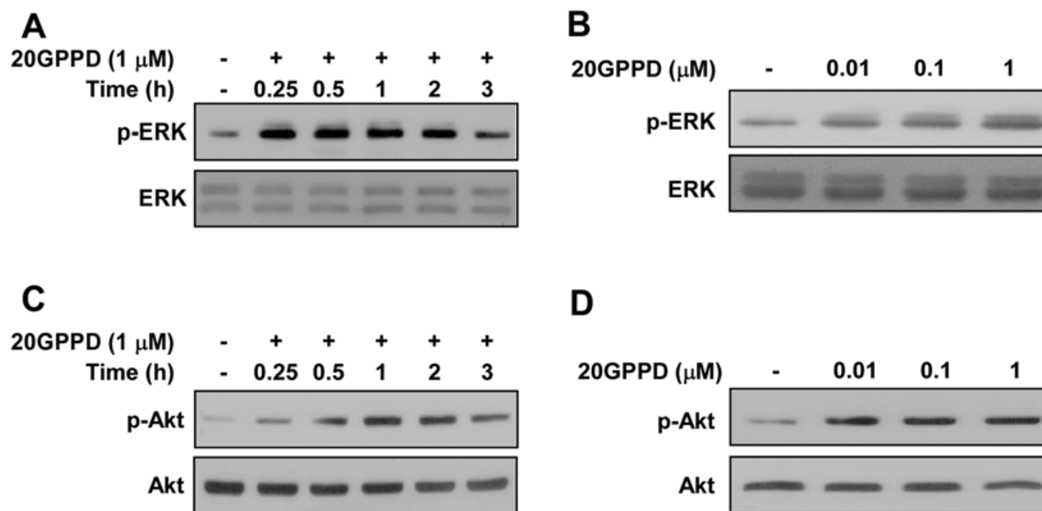


Figure 3. 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (20GPPD) induces the phosphorylation of extracellular signal-regulated kinase (ERK) and Akt in HaCaT cells. (A and B) 20GPPD induced ERK phosphorylation. (A) Cells were starved in serum-free medium and treated with 20GPPD (1 $\mu$M) for the indicated periods of time. (B) Cells were starved in serum-free medium and treated with the indicated concentrations of 20GPPD for 1 h. (C and D) 20GPPD induced Akt phosphorylation. (C) Cells were starved in serum-free medium and treated with 20GPPD (1 $\mu$M) for indicated periods of time. (D) Cells were starved in serum-free medium and treated with the indicated concentrations of 20GPPD for 1 h. The level of ERK, Akt, phosphorylated ERK (p-ERK) and phosphorylated Akt (p-Akt) was determined by immunoblot analysis as described in the Materials and methods.

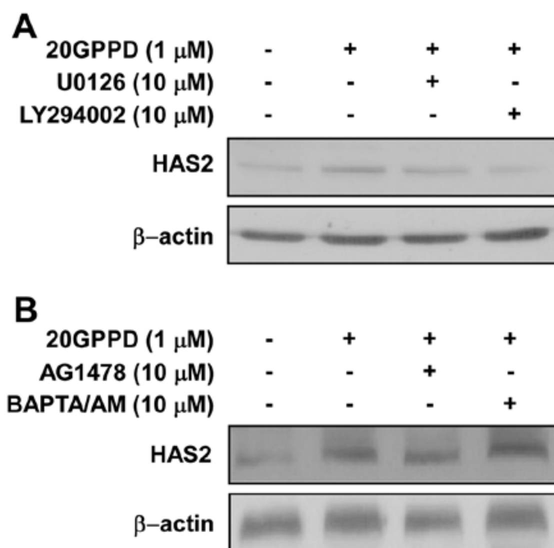


Figure 4. Extracellular signal-regulated kinase (ERK) and Akt mediate the 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (20GPPD)-induced hyaluronan synthase 2 (HAS2) expression. (A) Pharmacological inhibitors of MEK (U0126) and PI3K (LY294002) inhibited the 20GPPD-induced HAS2 expression. Cells were starved in serum-free medium and treated with U0126 and LY294002 1 h prior to treatment with 20GPPD for 3 h. (B) AG1478 (EGFR inhibitor) and BAPTA/AM (intracellular $Ca^{2+}$ chelator) did not affect the 20GPPD-induced expression of HAS2. Cells were starved in serum-free medium and treated with AG1478 and BAPTA/AM 1 h prior to the treatment of 20GPPD for 3 h. Immunoblot analysis was performed to measure the level of HAS2 and β-actin expression as described in the Materials and methods.

employed. When the cells were treated with these inhibitors 1 h prior to treatment with 20GPPD, the 20GPPD-induced expression of HAS2 was diminished in both the U0126 and LY294002 treatment groups (Fig. 4A). Certain studies have reported that HA synthesis is mediated by the EGF receptor (EGFR) (15,28,29) and $Ca^{2+}$-mediated signaling pathways, thus prompting us to investigate the effect of EGFR inhibition and intracellular $Ca^{2+}$ inhibition on the 20GPPD-induced HAS2 expression. However, neither AG1478 (an EGFR inhibitor) nor BAPTA-AM (an intracellular $Ca^{2+}$ chelator) affected the 20GPPD-induced expression of HAS2 (Fig. 4B). Overall, these results suggest that the 20GPPD-induced expression of HAS2 is mediated by the MEK and PI3K signaling pathways, rather than by EGFR or intracellular $Ca^{2+}$ signaling in HaCaT cells.

*Src regulates the 20GPPD-induced production of HA by inducing ERK and Akt phosphorylation*. It has previously been demonstrated that Src regulates HAS2 expression in human dermal fibroblasts (23). To further determine whether 20GPPD induces Src signaling in human keratinocytes, Src phosphorylation was evaluated. Src phosphorylation was observed to be increased following treatment with 20GPPD in a dose-dependent manner at 20 min (Fig. 5A). Furthermore, the role of Src in the 20GPPD-induced production of HA was investigated using PP2, an Src inhibitor. When the cells were treated with PPT for 1 h prior to treatment with 20GPPD, the 20GPPD-increased production of HA (Fig. 5B) was observed to be decreased along with HAS2 expression (Fig. 5C). It has been reported that Src is an upstream regulator of ERK and Akt signaling. Zhang *et al* reported that Src mediates breast cancer cell migration by activating Akt and ERK1/2 (30), while the inhibition of Src in vascular smooth muscle cells has been observed to suppress ERK and Akt phosphorylation (31). We thus investigated the effects of Src inhibition by PP2 on the 20GPPD-induced phosphorylation of ERK and Akt in the HaCaT cells (Fig. 5D). 20GPPD significantly induced ERK and Akt phosphorylation, whereas treatment with PP2 abolished both the phosphorylation of ERK and Akt in a dose-dependent manner. These results suggest that Src mediates the effects of 20GPPD on the production of HA by activating ERK and Akt signaling.

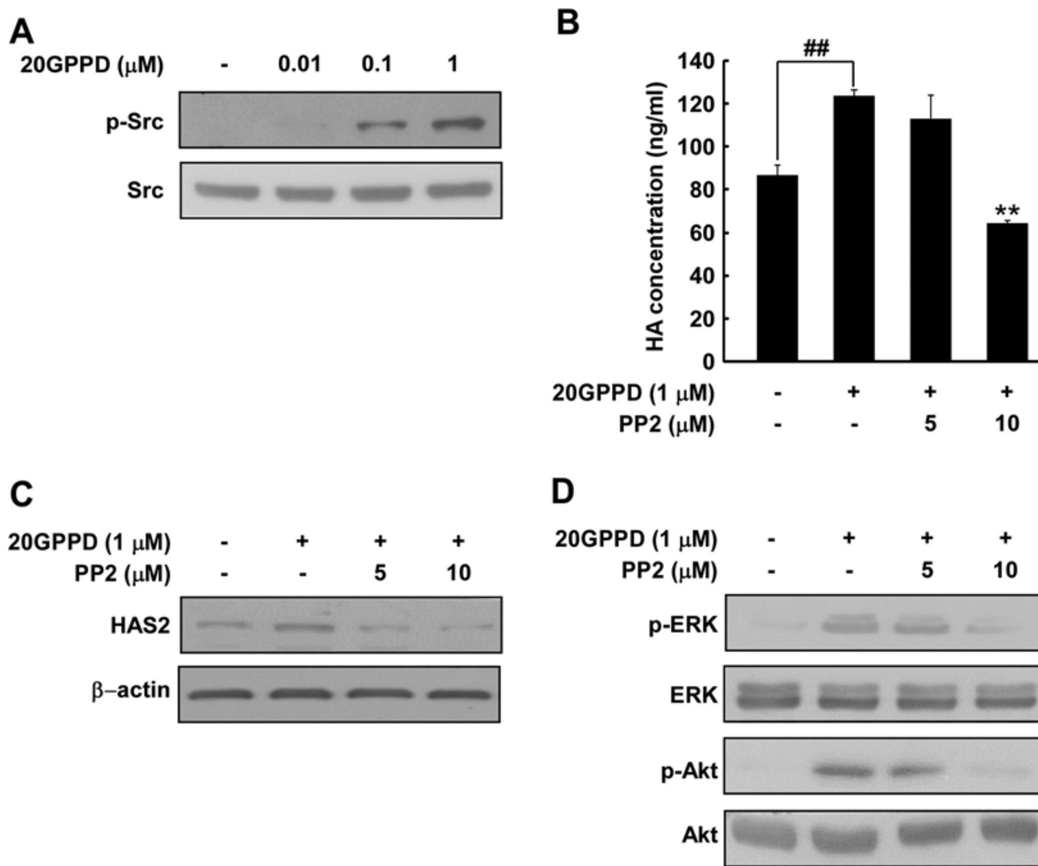


Figure 5. Src is the upstream regulator of extracellular signal-regulated kinase (ERK) and Akt in mediating the 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (20GPPD)-induced expression of hyaluronic acid (HA). (A) 20GPPD induced Src phosphorylation in HaCaT cells. Cells were starved in serum-free medium and treated with the indicated concentrations of 20GPPD for 20 min. (B and C) Cells were pre-treated with PP2 (Src inhibitor) for 1 h, and then exposed to 20GPPD for an additional 3 h. (B) HA concentrations were measured by enzyme-linked immunosorbent assay (ELISA) and (C) the expression of hyaluronan synthase 2 (HAS2) and β-actin was determined by immunoblot analysis. Data represent the means ± SD. ##P<0.01 vs. the untreated control; **P<0.01 vs. 20GPPD-treated group. (D) PP2 attenuated the 20GPPD-induced phosphorylation of ERK and Akt in HaCaT cells. Following pre-treatment with PP2 for 1 h, the cells were exposed to 1 μM 20GPPD for 1 h. Proteins were then subjected to immunoblot analysis to determine the level of ERK, phosphorylated ERK (p-ERK), Akt, and phosphorylated Akt (p-Akt) expression.

## Discussion

Increasing HA synthesis represents a promising strategy to improve skin hydration. HA is normally synthesized by one of 3 HAS enzymes; HAS1, HAS2 or HAS3. Tammi and Tammi (17), reported that HAS2 expression was reduced in the epidermis and dermis of intrinsically aged human skin. Therefore, the upregulation of HAS2 expression may represent a novel strategy for improving HA levels in the skin.

20GPPD is a major metabolite of ginsenoside Rb1, which has been shown to increase HAS2 mRNA expression and the HA content in human keratinocytes and increase epidermal thickness within the skin of a hairless mouse model (10). However, the mechanisms underlying these effects of 20GPPD remain unknown. In this study, we therefore examined the molecular mechanisms responsible for the enhancement of HA production by 20GPPD in HaCaT cells in an effort to verify its potential beneficial function in skin hydration. In a previous study, the bioavailability of 20GPPD was estimated using intravenous (i.v.) injection and oral administration in rats (32). The corresponding dose of 20GPPD (1 μM) was achieved in plasma concentration by both i.v. injection and oral administration. Furthermore, the authors demonstrated the rapid absorption of 20GPPD using Caco-2 cell permeability assay (32). Given that the highest dose of 20GPPD we used in this study was 1 μM, our results provide useful insight for the prediction of clinical outcomes.

Previous studies have demonstrated the upregulation of HAS through Akt and ERK (23). Our findings demonstrated that treatment with 20GPPD induced the phosphorylation of both ERK and Akt (Fig. 3A), but not JNK or p38 phosphorylation (unpublished data). Pharmacological inhibitors of ERK and Akt blocked the effects of 20GPPD on HAS2 expression, suggesting that ERK and Akt are the key players in 20GPPD-induced expression of HAS2. It has also been established that EGFR and $Ca^{2+}$ signaling plays a critical role in keratinocyte homeostasis (33-36). We thus employed AG1478 (an EGFR inhibitor) and BAPTA/AM (an intracellular $Ca^{2+}$ chelator) to examine the involvement of EGFR $Ca^{2+}$ signaling in the 20GPPD-increased expression of HAS2. However, neither AG1478 nor BAPTA/AM treatment affected the 20GPPD-induced expression of HAS. These results support our

hypothesis that the 20GPPD-induced expression of HAS2 is mediated by ERK and Akt, but not by EGFR or a $Ca^{2+}$-related signaling pathway.

Previous studies have indicated that Src mediates ERK and Akt signaling (37,38). In addition, Yang *et al* reported that Src and ERK enhance the HA oligosaccharide content in an endothelial cell model (39). In the present study, we demonstrated that Src mediated the 20GPPD-induced production of HA by activating ERK and Akt signaling. The increased HA content following treatment with 20GPPD was found to be abolished by PP2, an inhibitor of Src family kinase. In the group treated with 10 $\mu$M of PP2, the HA level was found to be lower than the level in the control group (Fig. 5B). This may be explained by the fact that PP2 is an inhibitor of several Src family kinases, rather than a specific Src inhibitor. As described above, studies have reported that Src is an upstream regulator of ERK and Akt (37,38). Indeed, we demonstrated that the 20GPPD-induced phosphorylation of ERK and Akt was decreased by the suppression of Src activity by PP2 (Fig. 5D). Therefore, we hypothesized that Src mediates ERK and Akt signaling during the 20GPPD-induced production of HA. It is notable that a previous study indicated that HAS2 can be regulated by *O*-linked *N*-acetylglucosamine (*O*-GlcNAcylation) on serine 221 (40). The effect of 20GPPD on this additional regulatory mechanism requires further investigation.

In conclusion, our data underline the potential skin hydrating effects of 20GPPD through the activation of HA production. Moreover, we found that Src tyrosine kinase is a key regulator of the 20GPPD-induced production of HA which occurs through the activation of ERK and Akt, but not through that of JNK or p38. To further support these findings, *in vivo* studies using an Src knockout model are warranted.

## Acknowledgements

This study was supported by the National Leap Research Program (no. 2010-0029233) through the National Research Foundation funded by the Ministry of Science, ICT and Future Planning, Republic of Korea and by the R&D program of MOTIE/KIAT (Establishment of Infra Structure for Anti-aging Industry Support; No. N0000697), Republic of Korea.

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